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Research Note—

Identification of *Pasteurella multocida* CHAPS-Soluble Outer Membrane Proteins

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SUMMARY. Fowl cholera continues to be of concern to the poultry industry, especially for turkey growers. This disease costs the turkey industry millions of dollars annually. In order to develop improved live attenuated vaccines or subunit vaccines, the outer-membrane proteins of *Pasteurella multocida* were examined with the use of proteomics. Of the 11 proteins total present in an outer-membrane subfraction of *P. multocida*, four additional proteins were identified, completing the composition of the detergent-soluble cross-protective protein fraction. These additional four proteins include protective bacterial surface antigen, OMA87 (Accession no. 15603857); heme–hemopexin receptor, HemR (Accession no. 15602441); lactate permease, LctP (Accession no. 15603717); and heptosyl transferase F, RfaF (Accession no. 15603709). Both the Oma87 and the HemR proteins would be of interest for subunit and modified live vaccine studies, respectively, because of their purported roles as virulence factors for *P. multocida*.

RESUMEN. *Nota de Investigación*—Identificación de las proteínas de la membrana externa de *Pasteurella multocida*.

El cólera aviar continúa siendo una preocupación para la industria avícola, especialmente para los criadores de pavos. Esta enfermedad cuesta a la industria de los pavos millones de dólares cada año. Con la finalidad de desarrollar mejores vacunas vivas atenuadas, se examinaron mediante análisis proteómicos las proteínas de la membrana externa de *P. multocida*. De las 11 proteínas totales presentes en la subfracción de la membrana externa de *P. multocida*, se identificaron cuatro proteínas adicionales, completando de esta forma la composición de esta fracción proteica soluble en detergentes y de protección cruzada. Estas cuatro proteínas adicionales incluyen el antígeno protectivo de superficie bacterial, denominado OMA87 (número de acceso 15603857); el receptor heme-hemopexina, identificado como HemR (número de acceso 15602441); las proteína lactato permeasa, LctP (número de acceso 15603717) y la proteína heptosyl tranferasa F, denominada RfaF (número de acceso 15603709). Debido a su supuesto papel como factores de virulencia para *P. multocida*, tanto la proteína Oma87 como la HemR pueden ser de interés para estudios de vacunas de subunidad y vacunas vivas modificadas, respectivamente.

Key words: *Pasteurella multocida*, outer-membrane protein, proteome, cross-protective protein fraction

Abbreviations: CHAPS = 3-([3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate; kDa = kilodalton; MALDI-TOF = matrix-assisted laser desorption/ionization time-of-flight; MOPS = 3-(N-morpholino)-2-hydroxypropanesulfonic acid; SDS-PAGE = sodium dodecyl polyacrylamide gel electrophoresis

Fowl cholera, a disease caused primarily by three serotypes of *Pasteurella multocida*, serotypes A:1, A:3, and A:4, costs the turkey industry millions of dollars annually. A live attenuated commercially available vaccine strain, strain M-9 (serotype 3,4), has been implicated in outbreaks associated with vaccination (2,3,11,17). Commercial bacterins prepared from serotypes A:1, A:3, and A:4 confer cross-protection and are currently used to control outbreaks of disease in poultry flocks (2,17). Research on the composition and identity of outer membrane proteins of *P. multocida* is continuing in order to identify one or more protein candidates for development of live avirulent, attenuated vaccines or subunit vaccines. Rimler's (16) work has focused on the development of a cross-protective subunit vaccine for fowl cholera. He identified a cross-protective *P. multocida* outer membrane-associated protein in a CHAPS-soluble fraction with a molecular mass of approximately 39 kDa. This protein was subsequently identified as the *Pasteurella multocida* lipoprotein B, PlpB (19). However, other proteins of the *P. multocida* outer membrane may also confer protection. Five of the 11 proteins present in Rimler's outer membrane fraction were identified (16,19). The present study describes the identity of the remaining outer-membrane proteins of the CHAPS-soluble preparation that could not be identified by the trypsin digestion method (19). Instead, we employed chymotrypsin for in-gel digestion of the protein bands and matrix-assisted time-of-flight mass spectrometry (MALDI-TOF) to identify the proteins.

MATERIALS AND METHODS

Outer-membrane proteins. The outer-membrane protein extract was prepared by Rimler (16) from *in vitro*–grown *P. multocida* serotype A:3, strain P-1059. Briefly, the bacterial pellet was suspended in a solution containing lysozyme, EDTA, and Triton X-100. The viscosity was reduced by incubating the suspension with DNase and hyaluronidase (15). The suspension was centrifuged at 100,000 × *g* for 1 hr. The supernatant was removed and the pellet was extracted with 0.5% 3-([3-cholamidopropyl]-dimethylammonio)-1-propanesulfonate (CHAPS). The extract was stored frozen at −80 C.

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). The CHAPS-solubilized proteins were denatured under reducing conditions (10) and applied to either a 10-well (10 μg protein per well) or a 2D well (250 μg protein) 4–12% Bis-Tris gradient gel (Invitrogen, Carlsbad, CA) with the use of the 3-(N-morpholino)-2-hydroxypropanesulfonic acid (MOPS) buffer system (Invitrogen). Gels were stained with Coomassie Brilliant Blue R-250 and destained as described (10).

Trypsin digestion and MALDI-TOF mass spectrometry. Gel plugs were removed from the stained bands of the gel by using a blunt-cut 16-gauge needle. The gel plugs were deposited in a 96-well microtiter plate

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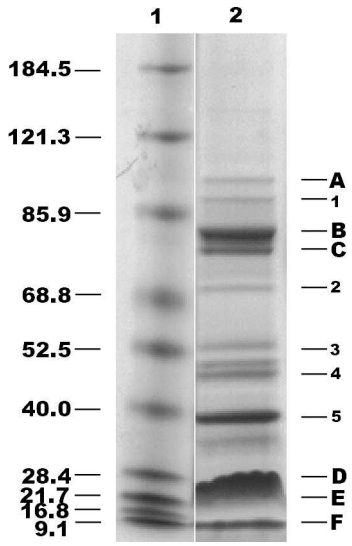

Fig. 1. SDS-PAGE of the CHAPS-soluble outer-membrane protein fraction. Lane 1, molecular weight markers indicated in kilodalton. Lane 2, CHAPS-soluble proteins. Bands A–F are described in Table 1. Bands 1–5 were previously identified (19).

and digested with chymotrypsin with the use of the automated digester, ProGest (Genomic Solutions, Ann Arbor, MI). The extracted peptides were desalted with the use of a C18-ZipTip (Millipore, Bedford, MA), mixed with an equal volume of saturated α-cyano-hydroxy-cinnamic acid (Sigma Chemical Co., St. Louis, MO) in 30% acetonitrile–0.2% trifluoroacetic acid and spotted onto the target plate. MALDI mass spectra were obtained by using a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA).

We used the MS-Fit search engine of the Prospector Web site of the University of California at San Francisco (http://prospector.ucsf.edu/) to search the nonredundant database of the National Center for Biotechnology Information (nrNCBI).

RESULTS AND DISCUSSION

SDS-PAGE analysis of protein bands A–F gave molecular weights of 104, 84, 77.5, 30.3, 17.5, and 15.1 kDa, respectively (Fig. 1). Bands 1–5 were previously identified (19) in the outer-membrane CHAPS-soluble preparation (16). The identities of the proteins A–F (Table 1) were obtained by peptide mass fingerprinting of the chymotrypsin peptide fragments rather than the trypsin peptide fragments. Two of the 6 proteins, the 104-kDa and the 17.5-kDa proteins, could not be identified even after repeated peptide mass analysis. Possible reasons include that only a small number of peptide fragments were generated with a monoisotopic mass greater than 900, and a relatively large number of peptides with a small monoisotopic mass of less than 600. Data such as these do not generate sufficient information to identify a protein with the MS-FIT algorithm (4).

Table 1. MS-FIT analysis of *Pasteurella multocida* outer membrane proteins A–F.

Band	Gel MW (kDa)	MW/pI (kDa/pH)	% Match	ID/Accession No.
A	104	ND[A]	–	–
B	84.0	87.5/7.6	45	OMA87/15603857[B]
C	77.5	80.7/9.4	42	HemR/15602441[C]
D	30.3	29.6/9.8	18	LctP/15603717[D]
E	17.5	ND[A]	–	–
F	15.4	17.7/9.6	13	RfaF/15603709[E]

[A]Not determined.
[B]Protective bacterial surface antigen by homology to *H. influenzae* D15 (Accession no. 16272854).
[C]Heme–hemopexin receptor protein.
[D]Lactate permease P.
[E]Heptosyl transferase F.

The proteins identified include two potential virulence factors, the protective bacterial surface antigen (Oma87) and the HemR protein, the heme–hemopexin receptor protein. The gene encoding the Oma87 protein of *P. multocida* has been cloned and the recombinant protein provided protection against a virulent challenge with the use of a mouse model (18). Another study tested a recombinant N-terminal fragment that did not provide protection in a chicken model for fowl cholera (13). However, the conserved domain of this protein resides in the C-terminal half of the protein, is a member of the pfam01103.12 family of proteins named Bac-surface-Ag, and is expressed by a large number of pathogens (12). Therefore it would be of interest to reinvestigate this protein as a potential vaccine antigen for fowl cholera.

The HemR protein is involved in binding hemopexin-bound heme from serum important for iron and porphyrin acquisition by a number of pathogens (5,20,21). HemR-expressing pathogens enable the pathogen to obtain iron from a large number of heme-containing proteins including hemoglobin, myoglobin, heme-albumin, and catalase (1), ensuring survival in the host. The HemR protein has not been previously isolated from *P. multocida*. However, a different heme-iron receptor (HasR, Accession no. 15603487) protein has been described for *P. multocida* A:3 in cattle as an immunodominant, presumably a *fur* (ferric uptake regulator) gene-dependent iron-regulated outer-membrane protein (14) as is the expression of the HemR protein (9). Two additional proteins that were identified, the lactate permease P (LctP) and heptosyl transferase F (RfaF), are involved in nutrient uptake and membrane biogenesis, respectively. The RfaF gene is nine open reading frames upstream from the LctP gene. Interestingly, a heptosyl transferase mutant of *P. multocida*, which produced a truncated lipopolysaccharide moiety was unable to cause fowl cholera in chicken (6,7,8). Although lipopolysaccharide and the capsule of *P. multocida* have been identified as key virulence factors, other surface structures, those not present or identified in Rimler's outer membrane fraction, may also contribute to virulence and pathogenesis.

The protein complement of the outer-membrane preparation of Rimler (16) that provided protection in turkeys against challenge inoculation with virulent *P. multocida* has now been identified with the exception of two proteins. Among the proteins previously identified were the putative cross-protective factor protein, *Pasteurella* lipoprotein B (PlpB), a putative virulence factor, and an enzyme involved in iron acquisition from heme, protoporphyrinogen oxidase, HemY (19).

In summary, with the use of chymotrypsin peptide mass fingerprinting we have identified the remaining proteins of the outer-membrane cross-protective fraction of Rimler. Of these additional proteins, the outer-membrane protective antigen (Oma87) and the heme–hemopexin (HemR) receptor protein would be of interest for the development of potential cross-protective vaccines for fowl cholera.

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